LOOKING INTO THE ENERGY LANDSCAPE OF MYOGLOBIN

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Looking into the energy landscape of myoglobin

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Using the haem group of myoglobin as a probe in optical experiments makes it possible to study its conformational fluctuations in real time. Results of these experiments can be directly interpreted in terms of the structure of the potential energy surface of the protein. The current view is that proteins have rough energy landscapes comprising a large number of minima which represent conformational substates, and that these substates are hierarchically organized. Here, we show that the energy landscape is characterized by a number of discrete distributions of barrier heights each representing a tier within a hierarchy of conformational substates. Furthermore, we provide evidence that the energy surface is self-similar and offer suggestions for a characterization of the protein fluctuations.

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Although proteins have well-defined tertiary structures they exhibit substantial structural indeterminism. This structural disorder is reflected in the fact that a protein can assume a large number of conformational substates (CS's)[1]. Different CS's represent the same coarse structure but differ on a microscopic level. Proteins also exhibit structural dynamics going from one CS to another. The CS's are separated by energy barriers that can be crossed by thermal activation. Because the distribution of energy barriers is very broad, the dynamics of proteins cover an extremely wide range of time scales. From a biochemical point of view, studying the conformational dynamics of proteins is important because their biological functions are often accompanied by conformational changes. One of the most important events in biology, protein folding, is a conformationally dynamic process by its very nature. On the other hand, physicists are interested in proteins as ideal model compounds for the dynamic behaviour of related systems such as glasses and spin glasses[2].

The energy landscape

Understanding the conformational dynamics of proteins requires a detailed knowledge of the structure of their potential energy landscapes. Because of the large number of atoms, and the various kinds of interactions that determine the energy of a particular structure, such knowledge cannot be obtained by direct calculation. Therefore, there is great need for experimental methods that give direct access to the potential energy surface as well as allow a productive interplay between experiments and model simulations. From a wide range of experimental and theoretical studies it is becoming increasingly clear that the energy landscape of a protein has a very specific structure[3]. Ansari *et al.* suggested that the energy landscape is hierarchically organized[4]. The idea is that every CS within a particular tier in the hierarchy is split into a number of sub-states which comprise the next tier of CS's. The average height of the energy barriers between CS's within one tier decreases for descending tiers. Experimental evidence suggests that a number of tiers exist for myoglobin[3,4].

Structural dynamics and spectral line broadening

The use of optical line narrowing techniques on haem proteins provides great potential for the study of protein dynamics, particularly in the low-temperature region[5,6]. Excellent reviews are available that explain in great detail the general principles behind these techniques as well as their particular merits for the study of structurally disordered systems[7–10].

The central idea of the experiments presented in this paper is that the haem serves as an ultrasensitive probe for structural rearrangements of the protein. There are many studies that clearly show that the resonance frequencies of the haem are strongly coupled to the conformation of the apoprotein[11,12]. Structural fluctuations of the protein cause fluctuations of the optical resonance frequencies of the haem. This is called spectral diffusion. Spectral diffusion causes a time dependence of the homogeneous linewidth, Γh, of an optical transition[13]. The time dependence of Γh is related to a distribution, $P(R)$, of rates, R, of fluctuating perturbers of the frequency of the chromophore[14,15]. These perturbers represent con-

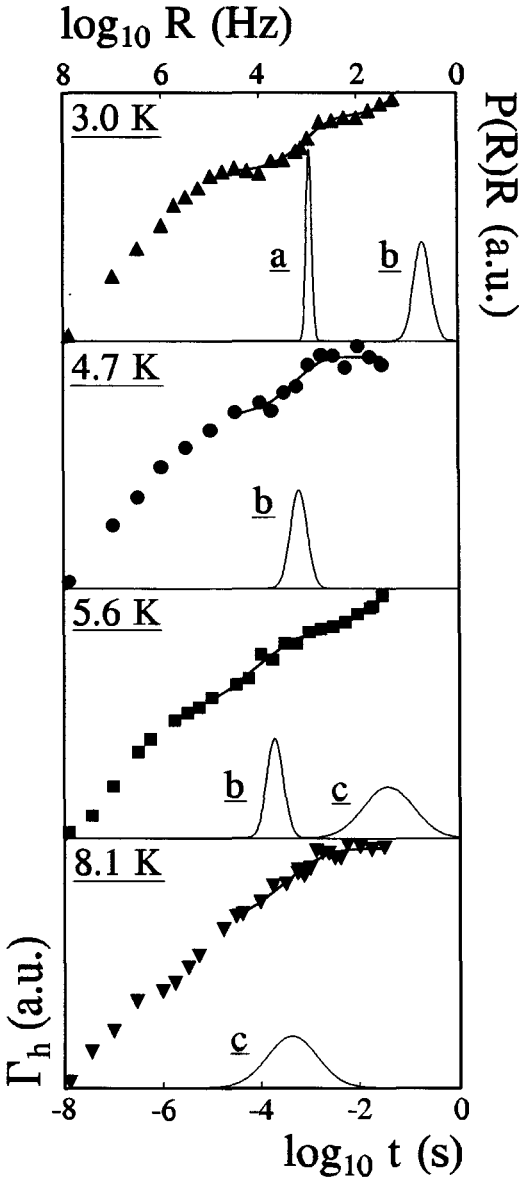

Fig. 1 Line broadening of the 0-0 transition of Zn-mesoporphyrin IX complexed to apomyoglobin as a function of time and temperature. The solid lines through the data are fits to Eq. 1 using the distributions that are displayed. See text for explanation.

formational fluctuations in the protein. Roughly speaking, fluctuations cause line broadening on a time scale of the inverse of their rates. The exact dependence is given by:

$$\Gamma_h(t) = \Gamma_h(t_0) + \int_0^\infty dR\, P(R) \{ \exp[-R t_0] - \exp[-Rt] \} \quad (1)$$

Our approach is straightforward. We measure the time evolution of Γ_h for the 0-0 transition of the Zn-substituted haem group in myoglobin. Data are subsequently fitted to Eq. 1 from which we obtain the distribution of rates at which the protein fluctuates between CS's. The temperature dependence of this rate distribution then gives specific information on the structure of the energy landscape.

Recently, we showed that below 3 K myoglobin exhibits conformational fluctuations described by a single narrow rate distribution[16]. Here, we focus on the issue of whether narrow rate distributions arise from a low density of CS's or from restrictions imposed by the structure of the potential energy surface.

The distribution of barrier heights

Fig. 1 shows Γ_h as a function of time at temperatures between 3.0 and 8.1 K. The most important information that can be deduced from these data is that the rate distribution that accounts for the time dependence of the line broadening consists of a number of discrete features that shift with temperature. Because each feature shifts with a different rate they move closer together with increasing temperature. As a consequence, individual features can only be resolved within a limited temperature range. From the data at 3.0 K it can be seen that there is a sharp feature, denoted as *a* (Fig. 1). From the broadening that starts at 10^{-3} s we can also see that a second feature, *b*, is 'moving in' from the direction of slow rates. At 4.7 K feature *b* has shifted enough to determine that it is also relatively sharp because the line broadening levels off in the millisecond-region. The same argument applies to feature c. At 5.6 K it 'moves in' while at 8.1 K we can see that the broadening levels off. Data at intermediate temperatures can be fitted using the same features, *a*, *b* and *c*.

An important issue is the mechanism by which transitions between CS's occur. At low temperatures, apart from classical thermally activated barrier crossing, tunnelling contributions may become important. A strong argument in favour of thermally activated barrier crossing arises from Fig. 2, which shows the temperature dependence of the fluctuation rates corresponding with the centres of the three discrete distributions that are shown in Fig. 1. The temperature dependencies of the rates corresponding with the three distributions each follow an Arrhenius law. The barrier heights and preexponentials obtained from linear fits to the data in Fig. 2 are presented in Table 1. If we accept thermal activation as the dominant mechanism, a picture of the potential energy surface emerges which is in excellent agreement with current views. In the case of a thermally activated mechanism each of the distributions of fluctuation rates corresponds to a distribution of barrier heights between CS's centred around the values given in Table 1. Each distribution of barriers corresponds to a single tier in a hierarchy of CS's. This explanation accounts for the narrowness of the distribution as well

Table 1 Thermodynamic parameters for conformational fluctuations of myoglobin

Rate distribution	a	b	c
E (kJ mol^{-1})	0.19	0.37	0.71
A (s^{-1})	2×10^6	7×10^6	2×10^7

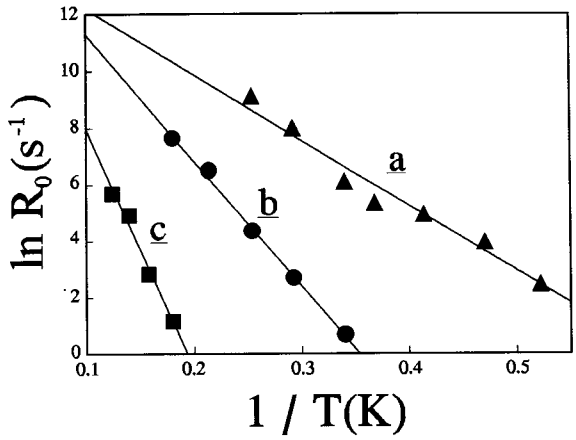

Fig. 2 Temperature dependence of the fluctuation rates, R_0, corresponding with the centres of the rate distributions obtained from line broadening data as displayed in Fig. 1. The triangles, circles and squares refer to the distributions in Fig.1 that were denoted as *a*, *b* and *c* respectively. The solid lines are fits to $R_0 = A \exp(-E/kT)$ with *A* the pre-exponential factor, *E* the barrier height and k Boltzmann's constant.

as the increasing barrier height corresponding with each successive distribution and the increasing magnitude of the line broadening associated with each successive distribution (*vide infra*). It should be stressed that this interpretation of the results in terms of the energy landscape of the protein relies on the assumption that thermal activation is indeed the dominant mechanism of the conformational fluctuations. However, we emphasize that, in the case of a tunnelling model, there is no plausible explanation for the regular behaviour that is observed.

Self-similarity?

In the remaining part of this paper we take the hierarchical model of CS's a little further and present a phenomenological model that seems to be in good agreement with the experimental observations. A closer look at the data reveals that the energy landscape may be more strictly ordered than is implied by a hierarchical organization of CS's. We observe that, moving upwards within the hierarchy, the average barrier height increases by the same factor of roughly two for successive tiers, while similar pre-exponentials are found. This suggests that the potential energy surface is self-similar in terms of the relation between barrier heights that characterize successive tiers. What we mean is that if a particular tier is characterized by an average barrier, E_i, then higher tiers exist with average barriers of αE_i, $\alpha^2 E_i$, ... and lower tiers exist with average barriers of $(1/\alpha)E_i$, $(1/\alpha^2)E_i$, ... There are indications that this apparent self-similarity covers more tiers of CS's than the three tiers that are deduced from Fig. 2. Extrapolation of the thermodynamic parameters displayed in Table 1 predicts that fluctuations within higher tiers are activated around 10 K, 20 K, 40 K and so forth. Evidence for such behaviour is given by temperature cycling hole burning experiments[17]. Evidence for the existence of lower tiers of CS's comes from the line broadening between 10^{-5} s and 10^{-8} s. Lower tiers will be characterized by barriers that are so small that even at very low temperatures the rates of barrier crossing are close to the upper limit determined by the preexponential factors. If all tiers are characterized by similar preexponentials, the rate distributions corresponding to the lower tiers will be too close together to be individually resolved. On the basis of the thermodynamic parameters given in Table 1, a self-similar model predicts that, above 3 K, line broadening induced by fluctuations within the lower tiers indeed occurs between 10^{-8} s and 10^{-5} s.

A microscopic picture

A self-similar model suggests that the protein fluctuations associated with each of the rate distributions belong to the same class of conformational rearrangements. The small preexponentials exclude the possibility of local fluctuations of small groups but point in the direction of global conformational fluctuations, although again we emphasize that this conclusion only holds for thermally activated barrier crossing. The data reveal that the magnitude of the total line broadening induced by the fluctuations that are characterized by the individual rate distributions increases for ascending tiers. The magnitude of the line broadening is a measure for the amplitude of the conformational fluctuations. This reflects the fact that fluctuations with larger amplitudes cause fluctuations of the optical frequency of the haem with a larger magnitude. If the conformational rearrangements are global this suggests that the mean atomic displacements between CS's increase for ascending tiers. In this case discrete distributions of barriers correspond to discrete distributions of mean atomic displacements between CS's. This also means that the magnitude of the spectral shifts of the haem that accompany transitions between CS's are restricted to certain values. For higher tiers the

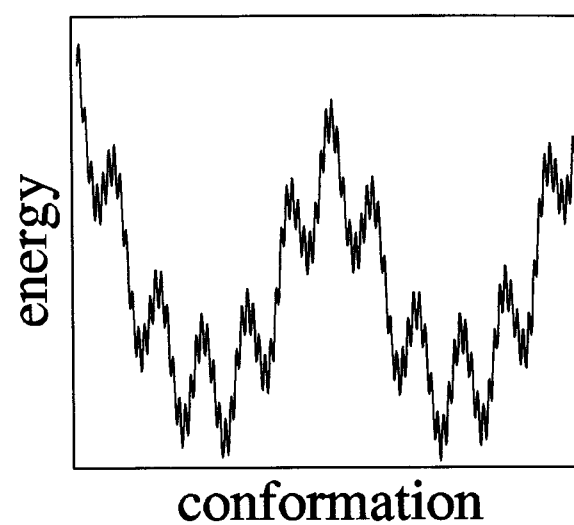

Fig. 3 Schematic representation of the potential energy surface of myoglobin based on the results presented. This representation is simplified in the sense that the true hyper-surface spans the coordinates of all atoms in the protein.

magnitude of the spectral jumps may become large enough to cause changes in the homogeneous line shape. Again this is observed in hole burning experiments[18].

The concepts provided in the previous discussion are united in Fig. 3 which gives a schematic representation of the potential energy surface.

Implications

It appears that, due to their highly specific nature, the results presented here may prove to be a serious test case for theoretical models that describe conformational dynamics of proteins, for instance, those involved in protein folding. In general terms the results quite explicitly show a fascinating property of proteins that seems to set them apart from related disordered systems such as glasses, spin glasses and polymers. The observation of substantial line broadening reveals that proteins are disordered, even at very low temperatures. However, the particular time- and temperature-dependent features show that the disorder itself is in fact ordered, as is shown by the highly organized structure of the energy landscape.

Methods

Sample preparation. The haem group of myoglobin from horse heart (Sigma) was extracted at pH 3.9 with 2-butanone. After several dialysis steps against water the apoprotein was reconstituted at pH 8.6 with Zn-mesoporphyrin IX (Porphyrin products) dissolved in dimethylformamide. The Zn-substituted protein was dialysed several times against 10 mM phosphate buffer pH 7.0. The volume was quadrupled with glycerol. After degassing samples were sealed in an cuvette of 1 mm optical path length. The protein concentration was approximately 1 mM. Samples were pre-cooled by plunging them into liquid nitrogen and further cooled to the desired temperature in a helium bath cryostat (Oxford instruments) equipped with an active temperature controller (Oxford instruments).

Spectroscopy. The homogeneous line width of the 0-0 transition of the Zn-mesoporphyrin was measured at 578 nm using three-pulse stimulated photon echoes. Principles and experimental details of the experiments are available elsewhere[16,19].

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